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Mass Fragmentographic Determination of Timolol in Human Plasma and Urine

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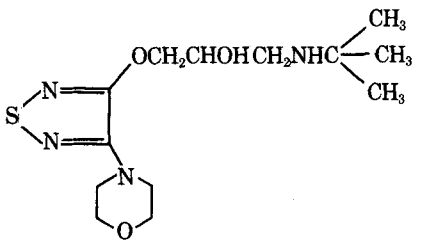
Abstract □ A mass fragmentographic procedure was developed for measuring quantities of <1.0 ng of timolol/ml of plasma or urine. The lower limit of sensitivity was 0.5 ng of timolol maleate/ml of plasma. The unchanged drug was extracted into heptane–4% isopentyl alcohol from alkalinized plasma or urine, together with propranolol hydrochloride as the internal standard. The compounds were subsequently back-extracted into 0.1 *N* HCl and then into chloroform following adjustment of the acidic phase to an alkaline pH. The chloroform layer was evaporated to dryness, and the compounds were derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide–acetonitrile to form the trimethylsilyl derivatives; these derivatives were quantitated by mass fragmentography. Recovery of timolol added to normal plasma and urine was quantitative and reproducible, and no interfering substances were observed in normal biological samples. After a 20-mg oral dose of timolol maleate, plasma levels of ~3.0 ng/ml were observed at 12 hr.

Keyphrases □ Timolol—extraction, derivatization, mass fragmentographic determination, plasma and urine □ β-Adrenergic blocking agents—timolol, extraction, derivatization, mass fragmentographic determination, plasma and urine □ Mass fragmentography—determination, timolol, plasma and urine

Timolol maleate, (−)-1-(*tert*-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol maleate, is a β-adrenergic receptor blocking agent with a high β-adrenergic inhibitor capacity. Due to the intensity of its effect, it is administered orally in small doses, varying between 10 and 20 mg, depending on the treatment needs. In addition, its high extravascular diffusion rate gives it an average apparent volume of distribution of 3.64 liters/kg. A low administered dose and a high apparent volume of distribution contribute to low plasma concentrations. For a proper pharmacokinetic study, assay techniques must be capable of measuring plasma levels as low as 0.5 ng of timolol/ml.

An electron-capture GLC determination of timolol in human plasma and urine was described by Tocco *et al.* (1). The lower limit of sensitivity was 2.0 ng/ml.

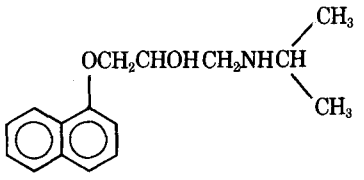
This report describes a mass fragmentographic procedure whose sensitivity threshold of 0.5 ng of timolol/ml of plasma enables plasma concentrations to be monitored for over 12 hr after oral administration of a 20-mg single dose of timolol maleate in a healthy adult.



I

EXPERIMENTAL

Reagents—Timolol (I) was used as the maleate salt[1], and propranolol (II) hydrochloride[2] served as the internal standard. All concentrations were expressed in terms of the base salt. Pesticide quality *n*-heptane[3], isopentyl alcohol[3], methylene chloride[4], ethyl acetate[5], and acetonitrile[6] nanograde reagent were used without further purification. Hydrochloric acid, sodium hydroxide, and double-distilled water were used in the preparation of 0.1 *N* HCl and 1.0 and 2.0 *N* NaOH. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide[7] served as the reagent for preparing the trimethylsilyl derivatives of timolol and propranolol.



II

[1] Merck Sharp & Dohme, Chibret, Paris, France.
[2] I.C.I., Enghien Les Bains, France.
[3] Prolabo R.P., Paris, France.
[4] Merck, Interchim, Montluçon, France.
[5] Fluka A.G., Interchim, Montluçon, France.
[6] Mallinckrodt, Interchim, Montluçon, France.
[7] Pierce Chemical Co., Interchim, Montluçon, France.

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Table I—Repeatability Assay with Timolol Maleate Plasma Samples

Samples with 1 ng/ml	Found, ng/ml
1	0.77
2	0.79
3	0.82
4	0.98
5	1.05
6	1.27
7	0.93
8	1.11
9	0.93
10	0.98
Mean ± *RSD*	0.96 ± 0.15

Table II—Repeatability Assay with Timolol Maleate Plasma Samples

Samples with 50 ng/ml	Found, ng/ml
1	47.2
2	50.4
3	51.7
4	54.0
5	49.8
6	48.1
7	50.2
8	52.3
9	48.7
10	51.3
Mean ± *RSD*	50.4 ± 2.1

Apparatus—The samples were analyzed on a GLC–mass spectrometer equipped for mass fragmentography[8] (M.I.D.). The gas chromatograph[9] was equipped with a 20-m × 0.03-cm glass capillary column with 2% OV1[10]. The gas chromatograph was operated isothermally with oven and injection temperatures maintained at 230 and 250°, respectively. The helium carrier gas flow rate was 10 ml/min. Other operating conditions were: source temperature, 260°; separator temperature, 240°; electron-impact energy, 35 ev; and trap current, 100 μamp. The M.I.D. program[11] was used with a computer[12].

Assay—The extraction was similar to that described by Tocco *et al.* (1). In a 10-ml glass-stoppered centrifuge tube were placed 1.0 ml of plasma or 0.1 ml of urine, 50 μl of propranolol internal standard (stock solution, 1000 ng/ml of water), 0.1 ml of 1.0 *N* NaOH, and 7 ml of heptane–4% isopentyl alcohol. The mixture was shaken mechanically for 10 min and centrifuged. In a 10-ml glass-stoppered centrifuge tube were placed 1.0 ml of 0.1 *N* HCl and 6 ml of the organic phase.

After the tubes were shaken and centrifuged, the organic phase was discarded by aspiration. To the aqueous phase were added 0.2 ml of 2.0 *N* NaOH and 7.0 ml of methylene chloride. The samples were shaken for 10 min and centrifuged.The organic phase was removed carefully, and 6.0 ml was added to a 10-ml silanized glass-stoppered tube. The contents were evaporated to dryness in a 60° heating block under a nitrogen stream.

The residue was dissolved in 0.1 ml of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide–acetonitrile reagent (10% in acetonitrile), and the stoppered tube was heated at 50° for 45 min. The tubes were cooled to room temperature, and then the contents were evaporated to dryness at 60° using a nitrogen stream. The residue was dissolved in 0.050 ml of ethyl acetate, and 2 μl was chromatographed.

The retention times of derivatized timolol and derivatized propranolol (internal standard) were 410 and 340 sec, respectively. GLC–mass spectrometric analysis showed that the products of silylation were the monotrimethylsilyl derivatives formed with the alcohol function of the lateral chain.

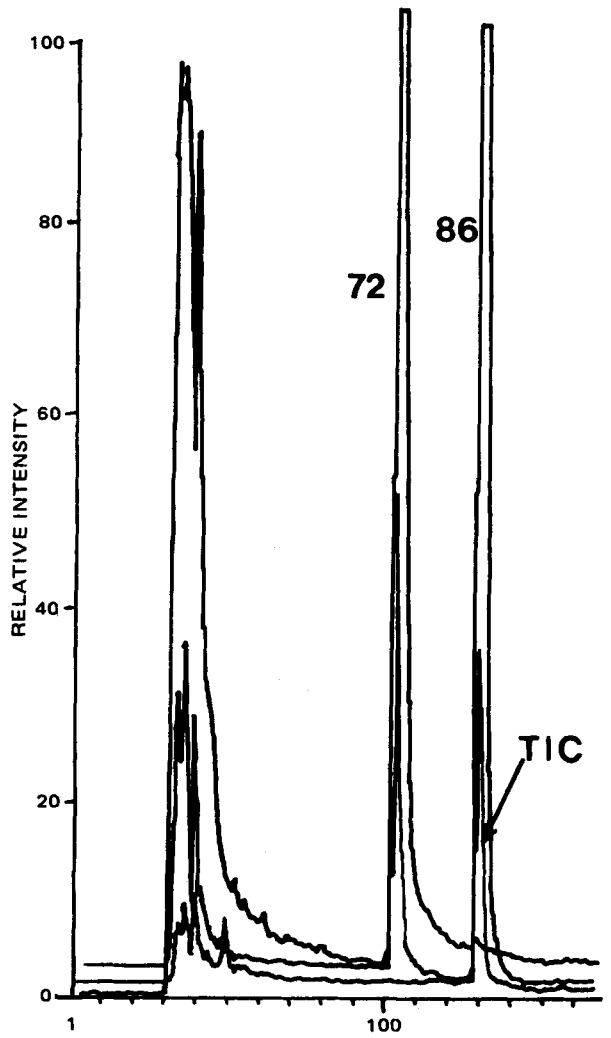

Figure 1—*Total ion current of a sample spiked with propranolol and timolol showing the traces of M − 72 and M − 86 fragments corresponding to the most abundant ions of trimethylsilyl derivations of propranolol and timolol.*

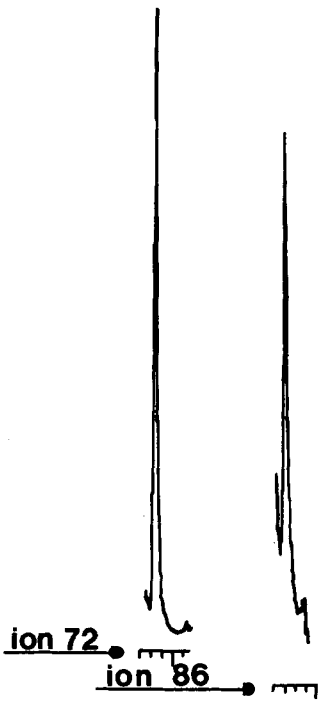

Figure 2—*Peaks obtained by mass fragmentography with plasma containing 50.0 ng of propranolol hydrochloride (amplification 20.0, m/z 72) and 1.0 ng of timolol maleate (amplification 150.0, m/z 86)/ml.*

[8] Model 2091 (electron impact, chemical ionization), L.K.B. Clinicon, Orsay, France.
[9] Pye Unicam Philips 204, Paris, France.
[10] Acta Co., Paris, France.
[11] Clinicon Co., Sweden.
[12] Digital PDP 11 with Tektronix 4012 and Central Printing Rank, Xerox, Paris, France.

Table III—Plasma Timolol Maleate Levels (Nanograms per Milliliter) in Five Human Subjects after a Single 20-mg Oral Dose of Timolol Maleate

Hours after Single Dose	Subject 1	2	3	4	5	Mean ± *SEM*[a]
0.50	32.2	1.4	26.0	15.6	108.1	36.7 ± 18.6
1.00	50.1	37.1	20.4	67.3	52.8	45.5 ± 7.9
1.50	33.1	81.2	53.2	63.4	52.8	56.6 ± 7.9
1.75	19.4	84.2	22.8	98.4	69.8	58.9 ± 16.1
2.00	26.6	60.7	32.1	68.0	36.0	44.7 ± 8.2
3.00	13.0	103.1	22.4	59.1	37.7	47.1 ± 16.0
4.00	11.1	54.6	15.5	30.2	25.2	27.3 ± 7.6
6.00	5.00	27.3	8.8	18.7	17.1	15.4 ± 3.9
8.00	3.2	15.7	4.6	10.5	10.0	8.8 ± 5.0
12.00	0.8	7.2	1.4	3.8	2.7	3.2 ± 2.5

[a] $SEM = RSD/\sqrt{5}$.

The M − 72 and M − 86 fragments, which constituted the most intense fragments in the mass spectra of the trimethylsilyl derivatives of propranolol and timolol, respectively, were used for the mass fragmentographic determination of propranolol and timolol.

Two standard curves (0–10 and 10.0–100.0 ng/ml of plasma) were constructed by analysis of samples of control plasma containing known quantities of timolol and propranolol. The ratios obtained by dividing the intensities of ion 86 by ion 72 were expressed and plotted against the concentrations of timolol maleate, in nanograms per milliliter of plasma or urine. The regression line of the data corresponding to the experimental points was drawn through the origin. Concentrations of timolol maleate were determined by interpolation from the standard curves.

Timolol in Human Plasma—Five healthy human subjects (two women and three men) were given a single 20-mg oral dose of timolol maleate (two tablets[13] of 10 mg). Blood was collected in heparinized containers at intervals over 12 hr following the single dose. Plasma was separated by centrifugation and frozen until assayed.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram obtained with plasma containing 50 ng of the internal standard and 50 ng of timolol (timolol maleate)/ml using total ion current detection. These peaks correspond to ions *m/z* 72 and 86 that were chosen for mass fragmentographic determination.

Blank plasma or urine samples gave no interfering peaks on the chromatogram. Metabolites of timolol with an unaltered lateral chain could interfere with the timolol peak, but their retention times are different. Two calibration curves prepared from two ranges of plasma levels (0–10.0 and 0–100 ng/ml of a same pool of plasma) indicated that intensity ratios of ions *m/z* 86 and 72 were linear when plotted against the concentrations of timolol maleate.

The lower limit of sensitivity was <0.5 ng of timolol maleate/ml of plasma or urine. Figure 2 shows the run obtained with a human plasma sample when 1.0 ng of timolol maleate was added to 1.0 ml of plasma. This concentration of timolol gave, for *m/z* 86, a peak height of ~100 mm with an amplification value equal to 150. The upper limit of linearity was at least 100 ng/ml. The coefficients of correlation were 0.9804 and 0.9948 for the low and high calibration curves, respectively.

[13] Timacor, Laboratoires Merck Sharp & Dohme, Chibret, Paris, France.

Repeatability assays were performed throughout the experiment on two pools of human plasma samples containing 1 and 50 ng/ml (Tables I and II). Timolol maleate concentrations were determined by interpolation from the corresponding standard curves. The mean concentrations (±*RSD*) were 0.96 ± 0.15 and 50.4 ± 2.1 ng of timolol maleate/ml of plasma with the low and high concentration plasma samples, respectively.

The lower limit of sensitivity for plasma levels expressed as the timolol salt is approximately five times lower than that obtained by Tocco *et al.* (1) using electron-capture GLC.

The accuracy of the technique is good since the relative standard deviation scarcely exceeded 16.0 and 4.2% of the averages obtained in the repeatability tests performed on plasma samples containing 1 (Table I) and 50 ng of timolol maleate/ml of plasma (Table II). These figures reflect an accuracy greater than that obtained with GLC analysis (1, 2).

This mass fragmentographic method is presumed to be specific for determining the intact compound in plasma and urine. The same procedure was applied to numefous urine samples in pharmacokinetic studies.

Plasma timolol levels were determined in five healthy human subjects over 12 hr following a single 20-mg oral dose of timolol maleate (Table III). Mean plasma timolol levels were near 3.2 ng/ml (Table III). The sensitivity level enables good approximate measurement of the plasma concentrations throughout pharmacokinetic studies. The terminal half-life of timolol was 2.62 ± 0.38 hr, and peak plasma levels of 82.5 ng/ml occurred at 1.55 hr after drug administration.

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